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Possible origin of undetectable EPO in urine samples

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Introduction

Erythropoietin (EPO) is a native human glycoprotein hormone, which main physiological effect is the induction of erythrocytosis and the consequent improvement of blood oxygen-carrying capacity. Because an increase of the number of erythrocytes enhances athletic performances in endurance sports, the use of synthetic (recombinant) forms of EPO is prohibited by the World Anti-Doping Code. Currently, the routine-used test, based on isoelectric focusing (IEF) of urinary EPO in polyacrylamide gels followed by double-blotting [1,2], allows to distinguish between endogenous and recombinant EPOs. However, approximately 15 % of all EPO tests carried out in anti-doping laboratories yield undetectable EPO profiles. An EPO profile is considered undetectable if no endogenous or recombinant EPO can be detected in a sample using the classical IEF-based test. In order to determine the possible origins of undetectable EPO profiles in athletes' urine, data obtained from a large number of official anti-doping urine tests aimed at detecting recombinant EPO were analyzed. In addition to physiological parameters representing potential causes for lack of EPO detection, the possible usage of proteasic adulterants to evade doping detection was also considered.

Materials and Methods

All EPO analyses were performed using a classical EPO isoelectric focusing test [1]. The potential usage of exogenous proteases in urine was screened using a Western blotting based method targeting some of the tryspsin-digested urinary albumin fragments.

Results and Discussion

Statistical analyses indicated that EPO undetectability in urine can be explained by at least two physiological characteristics: low EPO concentrations in the sample and very low OR very high urine specific gravities. More interestingly, the addition of very small quantities of protease in urine was shown to remove all traces of EPOs. This finding led to the development of a simple, specific and sensitive test based on albumin digestion that reveals proteasic activity.

In carrying out this work, it was aimed to highlight the causes of the high percentage of undetectable EPO profiles resulting from the classical anti-doping test. As expected, it was demonstrated that urine physiological characteristics clearly affect the detectability of an EPO profile. Additionally, in order to substantiate the rumors circulating among top level endurance athletes about adulterants that can alter the EPO test, potential exogenous causes were also considered. To this aim, a model based on trypsin, a very common protease, was developed to illustrate protein degradation. As simple addition of minute amounts of protease can also lead to undetectable EPO profiles, a cheating athlete could thus easily dissimulate a minuscule amount of protease powder and add it to his or her urine sample at some point during the collection procedure. Using the proposed test, an urine sample could then be rapidly analyzed for possible protease adulteration and the protease identified based on the observed albumin patterns without having to isolate the enzyme itself. Such an approach may prove extremely useful since proteases in solution are known to disappear overtime due to autolysis. At the same time, the use of Western blots as a means to detect polypeptides makes it possible to devise highly sensitive screening protocols requiring only small volumes of the urine samples. Finally, it was shown that such urine manipulation can be effectively countered by the addition to the sample of a concentrated cocktail of protease inhibitors, covering a large pH range. Therefore, this work has clear practical implications with regards to the improvement of the entire anti-doping control procedure.

Acknowledgements

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References

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[2] Lasne F. (2003) Double-blotting: a solution to the problem of nonspecific binding of secondary antibodies in immunoblotting procedures. J Immunol Methods 2003; 276:223-6.

Figures

Figure 1.

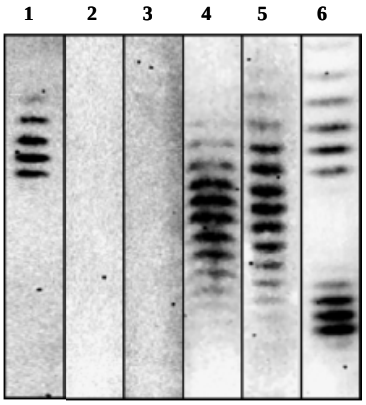

Fig 1: IEF gel of Trypsin-added urines with usual positive controls (Aranesp®/Recormon® standards, lane 6, BRP standard, lane 1). Lane 5 is a negative control. Lane 4 represents a negative control urine sample. Lane 3 represents this same negative control urine sample containing 50 mg/ml of Trypsin. Lane 2 represents a typical undetectable urine sample.

Figure 2.

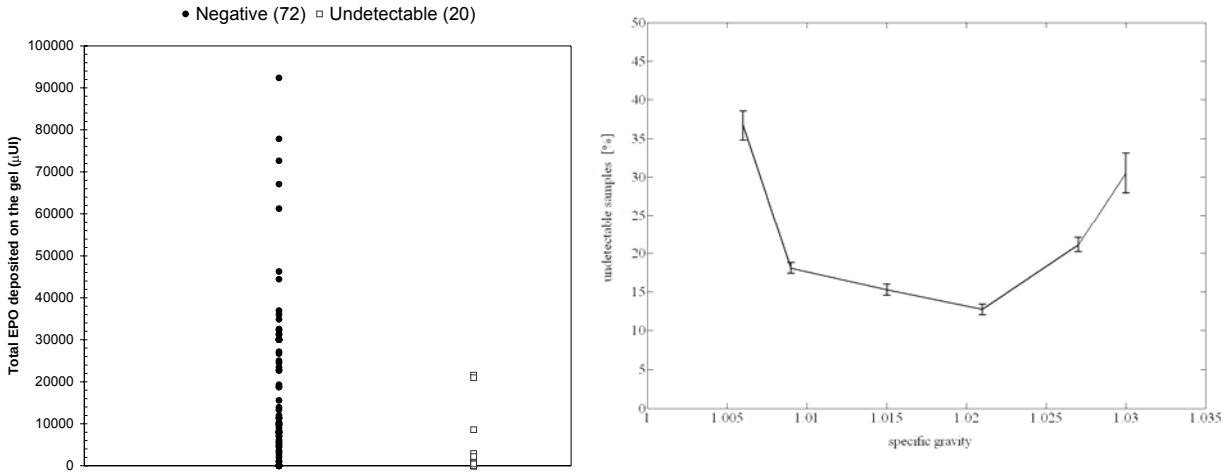

Fig 2 : A. Classification (detectable/undetectable) of 92 negative urine samples according to the total amount of EPO deposited on the gel. Note that all samples with the highest EPO concentrations (more than 25'000 mIU EPO deposited on the gel) were detectable. In contrast, all undetectable samples (N=20) had lower EPO concentrations, even if some samples with very low EPO concentrations were also detectable.
B. 2-by-2 representation of the specific gravity against the percentage of undetectable profiles, following the EPO analysis method described by Lasne. Undetectable profiles are mostly characterized either by very low or by very high urine specific gravities.

Figure 3.

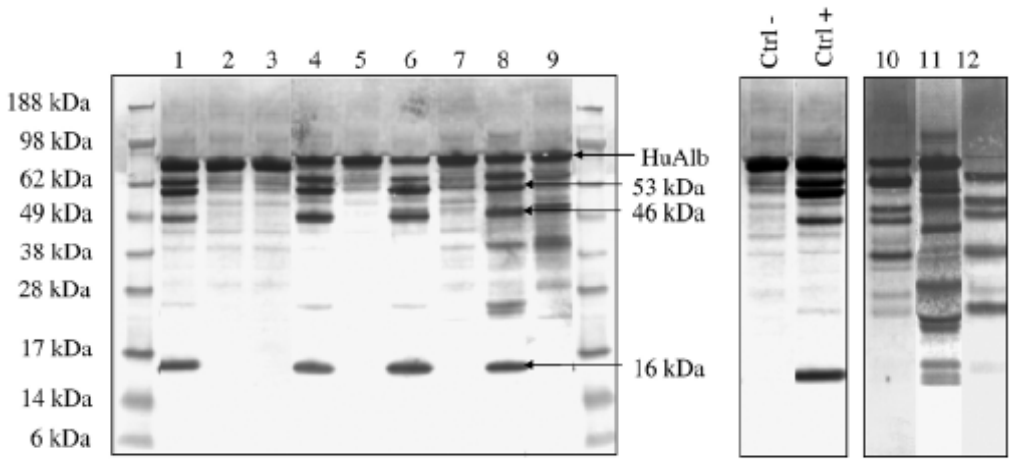

Fig 3 : Western blot analysis of Albumin content of various control urine samples and protease-added urine samples. Lanes 2, 5, 7 and 9 represent the control (non-spiked) urine samples of four different subjects. Lanes 1, 4, 6, 8 represent the corresponding Trypsin-added urine samples. Lane 3 represents a urine sample spiked with Trypsin and a protease inhibitors mix. Lane 10, 11 and 12 represent urine samples added with Protease from Streptomyces griseus type XIV, Papain, and Proteinase K at a concentration of 50 mg/ml, respectively. Ctrl – is human Albumin in physiological concentration. Ctrl + is Trypsin-added human Albumin.